US-CL-CURRENT: 442/76; 128/849, 128/888, 424/404, 442/123, 442/152, 442/153, 442/164, 442/79, 602/48, 602/50, 604/372, 604/374, 604/377

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | KWIC
Draw Desc | Image

☐ 8. Document ID: US 5728580 A

L3: Entry 8 of 11 File: USPT

US-PAT-NO: 5728580
DOCUMENT-IDENTIFIER: US 5728580 A

TITLE: Methods and culture media for inducing single cell suspension in insect cell lines

DATE-ISSUED: March 17, 1998

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
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US-CL-CURRENT: 435/348; 435/383, 435/384, 435/405

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | KWIC
Draw Desc | Image

☐ 9. Document ID: US 5705178 A

L3: Entry 9 of 11 File: USPT

US-PAT-NO: 5705178
DOCUMENT-IDENTIFIER: US 5705178 A

TITLE: Methods and compositions based on inhibition of cell invasion and fibrosis by anionic polymers

DATE-ISSUED: January 6, 1998

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Roufa; Dikla	St. Louis	MO		
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Frederickson; Robert C. A.	Cleveland	OH		
Coker, III; George T.	Mountain View	CA		

US-CL-CURRENT: 514/59; 514/54

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | KWIC
Draw Desc | Image

L3: Entry 8 of 11 File: USPT Mar 17, 1998

US-PAT-NO: 5728580
DOCUMENT-IDENTIFIER: US 5728580 A

TITLE: Methods and culture media for inducing single cell suspension in insect cell lines

DATE-ISSUED: March 17, 1998

US-CL-CURRENT: 435/348; 435/383, 435/384, 435/405

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L3: Entry 8 of 11 File: USPT

DOCUMENT-IDENTIFIER: US 5728580 A
TITLE: Methods and culture media for inducing single cell suspension in insect cell lines

Abstract Text (1):
Non-carboxylated sulfated polyanions have been successfully used to rapidly obtain and maintain stable single-cell suspension of BTI-TN5B1-4 cells, a cell line which has a high intrinsic capacity for the expression of recombinant protein, but which clumps severely in suspension reducing its effectiveness as a host for foreign protein production with the baculovirus expression vector system. The three most effective polyanions for inducing a single-cell suspension were dextran sulfate, polyvinyl sulfate, and pentosan sulfate. The cost of dextran sulfate treatment is low compared to heparin treatment, which required a 20-fold higher lever to induce single-cell suspension. More importantly, dextran sulfate does not block vital infection at MOI.gtoreq.1 whereas heparin is known to seriously inhibit infection. To overcome this effect, the cells can be subcultured to a fresh culture medium without the sulfated polyanion, or the sulfated polyanion is neutralized with a polycation, then the cells are inoculated with the baculovirus. Once the cells are infected with the baculovirus, the sulfate polyanion is added back to the fresh culture medium. Inducing single-cell suspension with dextran sulfate, a highly sulfated polyanion, resulted in an increased volumetric yield of recombinant protein. Examples chosen for experimentation included, human secreted alkaline phosphatase, and b-galactosidase. Optimum volumetric yield of 6 to 11 fold higher than attached cells for the production of alkaline phosphatase in BTI-TN5B1-4 dextran sulfate cells under elevated oxygen. More importantly, cells can be infected at high density without complications from aggregation, which has important implications for scale-up.

Brief Summary Text (2):
The invention pertains to the field of propagation of cell lines. More particularly, the invention pertains to methods of propagating cells in suspension.

Brief Summary Text (8):
Cell lines derived from Trichoplusia ni are difficult to grow in suspension. Wu et al. reported poor cell growth for TN368 cells in shake flasks. (Wu, J., King, G., Daugulis, A. J., Faulkner, P., Bone, D. H., Goosen, M. F. A. 1990. Adaptation of insect cells to suspension culture. J. Ferment. Bioeng. 70: 90-93.) Ogonah et al. found that TN368 cells grown in spinner flasks can be maintained for only 30-50 days and protein production was erratic. (Ogonah, O., Shuler, M. L., Granados, R. R. 1991. Protein production (.beta.-galactosidase) from a baculovirus vector in Spodoptera frugiperda and Trichoplusia ni cells in suspension culture. Biotech. Lett. 13: 265-270.) Hink and Strauss alleviated clumping of TN368 (another Trichoplusia ni cell line) cells by adding methylcellulose, but could not eliminate clumping completely. (Hink, W. F., Strauss, E. M. 1976. Growth of Trichoplusia ni (Tn368) cell line in suspension culture, pp. 297-300. In: E. Kurstak and K. Maramorosch (ed.), Invertebrate tissue culture, Academic Press, New York.) Chalmers et al. reported that cell damage occurs at the shear stress required to induce single-cell formation of TN368 cells. (Chalmers, J. J. 1995. The effect of hydrodynamic forces on insect cells, 175-204. In: M. L. Shuler, H. A. Wood, R. R. Granados and D. A. Hammer (ed), Baculovirus expression systems and biopesticides, Wiley-Liss, New York.) Taticek selected for single cells from suspension culture of TN5B1-4 but these cells eventually aggregated severely. (Taticek, R. 1995. Enhanced

recombinant protein expression in baculovirus-infected high-density insect cell suspension cultures and the operation of a continuous flow bioreactor. Ph.D. thesis, Cornell University, Ithaca, N.Y.) Taticek also used higher agitation speed in spinner flasks (ca. 160 rpm) which reduced aggregation but could not maintain good cell growth after 20-30 passages.

Brief Summary Text (9):
Reports on attempts to scale-up protein production in TN5B1-4 cells are limited, because of the problems associated with growing these cells in suspension. Overton and Kost used suspension culture of TN5B1-4 in a 5-L airlift fermentor to produce the secreted protein tissue inhibitor of metalloproteinases, but the volumetric yield was not significantly higher than adherent cultures. (Overton, L. K., Kost, T. A. 1995. Potential application of insect cell-based expression systems in the bio/pharmaceutical industry, 233-242. In: M. L. Shuler, H. A. Wood, R. R. Granados and D. A. Hammer (ed), Baculovirus expression systems and biopesticides, Wiley-Liss, New York.) Wickham and Nemerow used roller bottles coated with DEAE-based microcarriers to scale-up attached cultures to 5 L and obtained yields of 5-26 mg/L of soluble tissue factor. (Wickham, T. J., Nemerow, G. R. 1993. Optimization of growth methods and recombinant protein production in BTI-Tn5B1-4 insect cells using the baculovirus expression system. Biotechnol. Prog. 9: 25-30.) Chung et al. proposed using a split-flow, air-lift reactor with cells attached to glass beads in the downcomer section, and reported a volumetric yield of 10.7 mg/L of secreted alkaline phosphatase for a 0.5-L unit. (Chung, I. S., Taticek, R. A., Shuler, M. L. 1993. Production of human alkaline phosphatase, a secreted, glycosylated protein, from a baculovirus expression system and the attachment-dependent cell line Trichoplusia ni BTI-Tn5B1-4 using a split-flow air-lift bioreactor. Biotechnol. Prog. 9: 675-678.) Other scale-up strategies in attached cultures have been less successful. For example, TN5B1-4 cells do not grow well in roller bottles, and induce clumping of DEAE-based microcarrier beads by cell-cell bridging.

Brief Summary Text (13):
The use of heparin, a sulfated and carboxylated polysaccharide, has been reported to eliminate clumping in suspension culture of TN5B1-4 cells and was the subject of U.S. Pat. No. 5,348,877. However, heparin is expensive and inhibits viral infection resulting in poor protein production due to significant cell growth during infection which competes for nutrients. Since heparin had already been shown to induce single cell formation in TN5B1-4 cells, an effort was made to determine what structural features of heparin are important, which would hopefully lead to the identification of more effective dispersing agents that are potentially less inhibitory to viral infection.

Brief Summary Text (14):
The present inventors have successfully identified low-cost compounds that effectively eliminate clumping of insect cells in suspension culture. Specifically, the inventors have discovered that dextran sulfate and other sulfated polyanions can be used to increase protein production in the insect cell baculovirus system where a vital vector is involved.

Brief Summary Text (17):
Pat. Nos. 5,132,223 and 4,994,387 disclosing a medium containing dextran sulfate to replace heparin for the cultivation of human endothelial cells. In these patents, dextran sulfate was used to enhance cell growth and prolong the lifespan of endothelial cells. These patents are not relevant to the use of dextran sulfate or sulfated polyanions for inducing single cell suspension of insect cells. In particular, endothelials cells are obligate anchorage-dependent cells, ie., they grow only when attached to surfaces. In fact, because endothelial cells do not grow in suspension, the word "suspension" was not used in these patents.

Brief Summary Text (18):
U.S. Pat. No. 4,786,599 discloses a serum-free animal cell medium and method, for the primary culture and production of cell lines using the medium. The patent discloses the use of lipophilic biopolymers such as dextran for the dissolution of fatty acids. Dextran and Pluronic F68, two polymers commonly used to dissolve fatty acids, do not induce single-cell suspension of Tn5B1-4 cells. This patent does not disclose the use of sulfated polyanions to induce single-cell suspension or

enhancement of protein production.

Brief Summary Text (19):
U.S. Pat. No. 4,786,599 discloses a serum-free animal cell medium and method, for the primary culture and production of cell lines using the medium. The patent discloses the use of lipophilic biopolymers such as dextran for the dissolution of fatty acids. Dextran and Pluronic F68, two polymers commonly used to dissolve fatty acids, do not induce single-cell suspension of Tn5B1-4 cells. This patent does not disclose the use of sulfated polyanions to induce single-cell suspension or enhancement of protein production.

Brief Summary Text (20):
U.S. Pat. No. 5,024,947 discloses a serum-free media including a protective agent, such as dextran sulfate for the growth of insect cells and expression of products thereof. In this patent, protective agents are defined as "non-toxic, water soluble compounds that functionally act to protect insect cells from damage and death in agitated and sparged insect cell culture". In other words, protective agents are shear protectants. This patent does not teach that sulfated polyanions can induce single-cell suspension and enhance recombinant protein production. First, the two widely used protective agents, sodium CMC and Pluronic F68, do not induce single-cell suspension. Second, concentrations of 0.1% or greater are required for shear protection. In the case of dextran sulfate, the concentrations required for inducing single-cell suspension are not high enough to potentially exhibit any shear protection. At concentrations required for shear protection, dextran sulfate will be inhibitory to viral infection, i.e. protein production will be inhibited. In addition, our work indicates that dextran sulfate is toxic at concentrations greater than 0.1% so that it may become toxic before any shear protection is possible.

Brief Summary Text (21):
U.S. Pat. No. 5,348,877 teaches a method of adapting anchorage-dependent insect cell lines to suspension conditions using heparin. Heparin is not very effective in inducing single-cell suspension. A 20 fold higher heparin concentration is required relative to dextran sulfate. At concentrations required to induce single cell suspension, heparin inhibits viral infection resulting in poorer yield of recombinant protein compared to clumped cultures. This patent does not teach that a concomitant increase in protein yield is possible from inducing single-cell suspension. More importantly, this patent does not cover other sulfated polyanions such as dextran sulfate that can be used at much lower levels without inhibiting viral infection.

Brief Summary Text (22):
The use of non-carboxylated sulfated polyanions to induce single cell suspension in the insect cells is not described elsewhere in the literature. In mammalian cells, the results are not clear-cut. For example, heparin induces aggregation of lymphoid cells (Thurn and Underhill, 1986), and polyanions such a dextran sulfate induce aggregation of lymphocyte cells (Nakashima et al, 1993). On the other hand, high MW but not low MW dextran sulfate--such as that used in the examples disclosed herein--inhibited aggregation of human neutrophil cells (Rochon, et al, 1994). Also, dextran sulfate slightly inhibited aggregation of mouse cerebellar cells, but heparin and fucoidan sulfate had no effect (Fisher and Schachner, 1988). In cases where inhibition of cell aggregation was observed, the inhibition is not complete as the case with insect cells. More importantly, there is no data in the literature correlating inhibition of aggregation with increase in recombinant protein yield. Inducing single cells into suspension with polyanions to increase protein yield is not known in the art.

Brief Summary Text (23):
An important consideration for some of the sulfated polyanions is cost. For example, dextran sulfate costs only 3 cents/L of medium compared to $26/L for heparin. Since a lot of the research labs using insect cells to make recombinant proteins have shifted to Tn5B1-4 cells, there is a market for media that can be used to rapidly obtain and maintain stable single-cell suspension of T. ni cells.

Brief Summary Text (25):
The present invention teaches using sulfated polyanions to rapidly induce and

maintain single-cell suspension of TN5B1-4 cells which resulted in significant enhancement of recombinant protein production. A sulfated polyanion is added to the medium of an anchorage-dependent cell line. The cells which are in suspension after the addition of the sulfated polyanion are subcultured to a fresh culture medium including the sulfated polyanion.

Brief Summary Text (26):
This method has been shown to work with cell lines derived from the Cabbage Looper, Trichoplusia ni. Specifically, the normally anchorage-dependent cell line BTI-Tn-5B1-4, ATCC CRL 10859, was successfully induced to suspension conditions in serum free medium.

Brief Summary Text (28):
The three most effective polyanions for single-cell formation were dextran sulfate, polyvinyl sulfate, and pentosan sulfate - all highly sulfated. Dextran sulfate is the least expensive ($1.3/g) and increases medium costs by only 3 cents/L at 25 .mu.g/ml - not a significant cost upcharge with a medium that costs $5-30/L. Dextran sulfate works at a concentration of 25 .mu.g/ml. The cost of dextran sulfate treatment is low compared to heparin which required a 20-fold higher level to induce single-cell suspension and would add $26/L to medium costs. More importantly, dextran sulfate does not block viral infection at multiplicity of infection (MOI) greater than 1 plaque forming unit (PFU) per cell whereas heparin is known to seriously inhibit infection, an attribute shared by polyvinyl sulfate.

Brief Summary Text (29):
The present invention also teaches a method of culturing a cell line induced to single cell suspension by a sulfated polyanion that significantly inhibits infection by a baculovirus. The cells are subcultured to a fresh culture medium without the sulfated polyanion, or the sulfated polyanion is neutralized with a polycation, then the cells are inoculated with the baculovirus. Once the cells are infected with the baculovirus, the sulfate polyanion is added back to the fresh culture medium.

Brief Summary Text (30):
The intrinsic advantage of TN5B1-4 cells over the commonly used SF cell lines in per cell production can now be realized using single-cell suspension of TN5B1-4 cells which eliminates the problems that have so far limited the use of T. ni cells for large-scale production, including difficulty of growing T. ni cells in suspension, the poor yield in suspension, and the inefficiency of scaling-up attached cultures. The optimum volumetric yield of SEAP in TN5B1-4 DS 5000 cells under elevated oxygen but unsupplemented medium is 6 to 11-fold higher than attached cultures, and 3-fold higher than the best yield obtained for SF21 cells in suspension at elevated oxygen and with nutrient supplementation. The optimal per cell production of .beta.-galactosidase in TN5B1-4 cells was 350 U/ 10.sup.6 cells in unsupplemented medium compared to 140 U/ 10.sup.6 cells for SF21 cells with nutrient supplementation. The peak volumetric .beta.-galactosidase yield of TN5B1-4 cells at 2.times.10.sup.6 cells/ml in unsupplemented medium is lower compared to SF21 cells at 7.times.10.sup.6 cells/ml in supplemented medium (705 vs. 855 U/ml) probably because of nutrient limitations in TN5B1-4 cells as indicated by the rapid decline in per cell production with increasing density. By maintaining specific productivity with appropriate nutrient supplementation, TN5B1-4 cells should easily surpass the peak volumetric yield of .beta.-galactosidase in SF21 cells.

Brief Summary Text (31):
The intrinsic advantage in per cell production of TN5B 1-4 over the commonly used SF cell lines can now be realized in suspension culture by using inexpensive sulfated polyanions to rapidly induce and maintain single-cell suspension of TN5B1-4 cells which substantially enhanced protein production. High yields of up to 110 mg/L of the glycosylated, secreted protein human alkaline phosphatase and 2.3 g/L of the cytoplasmic protein .beta.-galactosidase have been obtained under elevated oxygen. Nutrient supplementation might potentially increase these yields further.

Detailed Description Text (2):
This description teaches the methods, materials and results of tests to determine whether various compounds sharing a structural feature(s) of heparin (polymer, sugar backbone, negative charge, sulfate groups, carboxylate groups, disaccharides,

monosaccharides) are effective agents for inducing single cell cultures of TN5B1-4 and if these cultures are more productive than cultures that contain clumps.

Detailed Description Text (3):
The present invention teaches that sulfated polyanions rapidly induce single-cell formation, which results in significantly enhanced protein production. Unsulfated polyanions, neutral polymers, polycations, disaccharides, and monosaccharides were not effective in inducing single cell populations. A sugar backbone was not essential for single-cell formation since a hydrocarbon backbone worked equally well. More importantly, two of the most effective polyanions, dextran sulfate and pentosan sulfate, do not significantly affect viral infection in contrast to heparin.

Detailed Description Text (5):
Heparin, dextran sulfate, fucoidan sulfate, pentosan sulfate, sodium carboxymethylcellulose, hyaluronate, chondroitin sulfate, dermatan sulfate, heparin disaccharides, chondroitin disaccharides, dextran, polyvinyl sulfate, l-carrageenan, k-carregeenan, sodium polyphosphoric acid, polyphosphate, glucose 6-phosphate, fructose 6-phosphate, protamine sulfate, DEAE-dextran (from dextran of MW 500,000), Pluronic F68, Antifoam C, heparinase I, heparinase II, chondroitinase ABC, hyaluronidase, 4' 6-Diamino-2-phenylindole (DAPI), fluorescein isothiocynate-dextran (FITC-dextran, MW 40,000), 5-bromo-4-chloro-3-indolyl b-D-galactopyranoside (X-gal), and 5-bromo-4-chloro-3-indolyl phosphate (BCIP), and rhodamine B isothiocyanate (RITC-dextran, MW 40,000) were obtained from Sigma Chemicals (St. Louis, Mo.). Dextran sulfate used for colloidal titration (MW 50000, 19% sulfur) was obtained from ICN (Costa Mesa, Calif.). Cat-Floc (polydiallyldimethyl ammonium chloride, MW 40,000) was a gift from Calgon Co. (Pittsburgh, Pa.). FITC-dextran sulfate (MW 40,000) was purchased from Molecular Probes (Junction City, Oreg.). DNase I, lipase, and Proteinase-K were from Boehringer Mannhelm (Indianapolis, Ind.). [Methyl-.sup.3 H]thymidine was obtained from Amersham Life Science (Arlington Heights, Ill.).

Detailed Description Text (8):
Suspension culture of TN5B1-4 cells was obtained from M. S. Donaldson (Cornell University). These cells were initiated from monolayer cells grown in TNM-FH medium (10% FBS) into spinner flasks at 160 rpm and adapted to the serum-free medium Ex-Cell 405 (JRH Biosciences, Lenexa, Kans.) by reducing the proportion of TNM-FH gradually until the cells were in Ex-Cell 405 after 4-6 passages. Cells formed clumps immediately when grown in suspension with over 50 percent of the cells in aggregates. The cells were subcultured without settling to avoid selecting for subclones. Hereafter, these cells are referred to as control cells.

Detailed Description Text (13):
Heparin is a natural mucopolysaccharide composed of D-glucuronic acid and D-glucosamine, and is fairly nonhomogeneous with respect to molecular weight and degree of sulfation. To explore the importance of various structural features on single-cell formation, we first considered dextran sulfate--a synthetic analog of heparin obtained by esterification of dextran. Dextran sulfate has no carboxylate group, but is highly sulfated with a maximum substitution of 3 sulfate groups per glucose residue. The degree of sulfation in commercial preparations of dextran sulfate is 2-2.5 groups per sugar unit.

Detailed Description Text (14):
Dextran sulfate induced single-cell formation from clumped TN5B 1-4 suspension grown in Ex-Cell 405, a serum-free medium. Most of the clumps were dispersed within 2 days and eliminated in 4 days. More severe level of clumping was eliminated in two passages: the first passage at high dextran sulfate concentration and the second at a much lower level. After inducing single-cell formation, a concentration of 25 .mu.g/ml of dextran sulfate 5000 was enough to maintain the single-cell population.

Detailed Description Text (17):
Clump formation in serum-free medium can be arrested by adding dextran sulfate when adapting monolayer cells to suspension. Cells adapted without dextran sulfate formed clumps after a few passages; clumping became more severe with time but eventually leveled off at passage 10 where typical aggregate sizes of 30-100 cells were observed. In contrast, cells grown in the presence of 25 .mu.g/ml dextran sulfate

remained as single cells. Cells in dextran sulfate were readily adapted to suspension culture and exhibited better growth than control cells. Enhanced cell growth in dextran sulfate may be due to better nutrient and oxygen transport to single cells compared to clumped cells.

Detailed Description Text (18):
A stable single cell suspension of TN5B1-4 was obtained from the clumped suspension culture by the addition of 25 .mu.g/ml of dextran sulfate (from dextran of MW 5,000; actual MW--12,500). The cells were passaged at least 5 times in medium containing dextran sulfate before use in experiments. Hereafter, these cells are referred to as DS 5000 cells.

Detailed Description Text (22):
TN5B1-4 cells grown in spinner flasks with dextran sulfate had a doubling time of 20 h, and reached a maximum density of 5-6.times.10.sup.6 cells/mi. Dextran sulfate 5000 does not affect the doubling time and the maximum cell density for levels between 25-600 mg/ml (dam not shown). The growth of TN5B1-4 DS 5000 cells in suspension was scaled-up to a 1.25 L Celligen reactor agitated with a marine impeller at 90 rpm. The cells were subcultured three times in this reactor with no clump formation. The doubling time was 20 h and a maximum density of 6-7.times.10.sup.6 cells/ml was reached.

Detailed Description Text (24):
The ability to induce single-cell suspension was tested with other compounds. These tests not only identify other agents that may be useful, but also provide information on what features of dextran sulfate contribute to its effectiveness.

Detailed Description Text (25):
Compounds were evaluated for their ability to induce single cell suspension in 50-ml spinner flasks at 160 rpm. Control cells were passaged at least 10 times in suspension before they were used in any experiments. Control cells were seeded at 5.times.10.sup.5 cells/ml, allowed to grow in the presence of the additive for 3 days, counted and observed in a microscope, and then subcultured at the same initial density. A qualitative score for reduction in clumping relative to control cells was assigned to cells grown in the presence of test compound at the end of the third passage. The protocol for enzyme treatment was the same, but the seeding density was 1.times.10.sup.6 cells/ml and cells were evaluated after 24 h. This data is summarized in Table 1 below.

Detailed Description Text (26):
The efficacy of dextran sulfate in inducing single-cell formation suggests that carboxylate groups of heparin are not important. Indeed, polysaccharides with only carboxylate groups such as sodium carboxymethylcellulose and hyaluronate did not induce single-cell formation (Table 1). The carboxylates of these macromolecules are ionized at the pH of the medium. Table 1 lists a number of polysaccharides with various degrees of sulfation and carboxylation and their effect in inducing single-cell formation. Two different molecular weight dextran sulfates have been included to bracket potential variations in efficacy due to size. The efficacy of inducing single-cell formation increased with the degree of sulfation.

Detailed Description Text (30):
The three most effective polyanions for single-cell formation were dextran sulfate, polyvinyl sulfate, and pentosan sulfate--all highly sulfated. For dextran sulfate and polyvinyl sulfate, 25 .mu.g/ml was adequate to maintain single-cell suspension whereas 100 .mu.g/ml of pentosan sulfate was necessary. At these concentrations, pentosan sulfate--and polyvinyl sulfate--treated cells grew with the same doubling time and reached the same maximum cell density as dextran sulfate cells and were grown up to 10 passages. TN5B1-4 has been maintained as single-cell suspension in spinner flasks for over 70 passages with 25 .mu.g/ml of dextran sulfate 5000, without significantly affecting doubling time, maximum cell density, and protein production.

Detailed Description Text (31):
TN5B1-4 cells have been maintained in single-cell suspension with dextran sulfate in a variety of serum-free media, including Ex-Cell 400, Ex-Cell 405, Express-Five, and

IPL 41-based media (personal communication, M. S. Donaldson). Inducing single-cell formation in serum-containing media was more difficult. Higher levels of dextran sulfate were required and although a substantial reduction in clumping was achieved, single-cell formation could not be induced. Serum may contain positively charged proteins which can neutralize dextran sulfate. The problem in serum-containing media is not a serious limitation since elimination of serum in suspension culture is desirable, because serum is expensive and comparable levels of recombinant proteins can be obtained in serum-free media.

Detailed Description Text (34):
Other studies have shown that sulfated polysaccharides induce aggregation of mammalian cells, indicating that these compounds have no intrinsic capacity to disaggregate cultures, and, in fact, disaggregation is an unexpected result. Thurn and Underhill found that heparin and dextran sulfate induced aggregation in cells of lymphoid origins but not in cell lines of fibroblastic origins. The ability of heparin to induce aggregation is closely related to the binding affinity of heparin to the cell surface. Conditions such as a shift in pH or ionic strength which lower the binding affinity of heparin increase aggregation. Nakashima et al. reported that polyanions such as dextran sulfate induce aggregation of mouse lymphocytes.

Detailed Description Text (46):
Approximately 27,000-30,000 DPM (disintegration per min) of [.sup.3 H]-labeled virus (.sup.- 300 virions/cell) was added to 1.4 ml of cell suspension or medium only containing the test compound, and incubated at 28.degree. C. The cell-free control was included to determine if the test compound induced virus precipitation. After 30 min incubation, the samples were transferred to microcentrifuge tubes and spun at 250.times.g for 2 min. A 0.7 ml aliquot of the supernatant was dissolved in scintillation fluid (Ready-Solv HP, Beckman Instrument, Fullerton, Calif.), and counted in a Beckman LS6800 scintillation counter which automatically converted counts per min to DPM using a resident quench curve obtained with tritium quench standards (Beckmann Instrument). Counting efficiency was over 40 percent.

Detailed Description Text (48):
The polymeric form was necessary for the binding inhibition since heparin, but not heparin disaccharides inhibited virus binding. Polycations at high concentration can induce virus precipitation such as the case with protamine sulfate. This precipitation is probably due to the bridging of the negatively-charged virus by the polycation, a phenomenon similar to the induced aggregation of charged liposomes by oppositely-charged polymers.

Detailed Description Text (49):
At concentrations required to maintain single-cell suspension of TN5B1-4, the three polyanions inhibited binding in the order dextran sulfate>pentosan sulfate=polyvinyl sulfate (Table 2). The binding of virus to TN5B 1-4 is very fast, with a rate constant that is about half of the maximum diffusion-limited binding. Dextran sulfate reduced the attachment rate constant six-fold, but binding was still significant, comparable to SF21 cultures without dextran sulfate. The binding of virus to TN5B1-4 in the presence of dextran sulfate and SF21 is reaction-limited.

Detailed Description Text (51):
The inhibition of binding was further explored by considering the effect of increasing polyanion concentration on the binding of virus to TN5B1-4 and SF21. Less than 20 .mu.g/ml of both dextran sulfate and heparin were required to attain maximum inhibition. For heparin, no additional drop in binding was observed for up to 600 .mu.g/ml. Dextran sulfate reduced the binding of the virus to TN5B1-4 but not to SF21. However, dextran sulfate inhibited vital infection in both cell lines, suggesting that inhibition of vital infection occurred at a step(s) subsequent to virus binding. The cell growth post-infection was used as a measure of virus infection since infection is known to inhibit cell division. Hereafter, synchronous infection is used to refer to infection with no measurable cell division post-infection.

Detailed Description Text (53):
Binding was not completely inhibited in the presence of increasing levels of heparin and dextran sulfate. Instead, a maximum level of inhibition was reached, with no

additional reduction in binding occurring beyond the threshold concentration. For heparin and dextran sulfate, the threshold value was less than 20 .mu.g/ml. One possible explanation for this behavior is that the polyanion is bound to the cell surface, and reached saturation at the threshold value. Since the same amount of polyanion is bound at concentrations above the threshold value, no additional reduction in binding is observed. An example of such occurrence is the binding of heparin to the lymphoid cell line YAA-C1, where binding is weak with a K.sub.d of 3.5.times.10.sup.-7 M, and the amount of bound heparin quickly reached saturation at 10 mg/ml (Thurn and Underhill, 1986). If sulfated polyanions reduce binding of baculovirus to TN5B1-4 by binding to the cell surface, the binding of the sulfated polyanions is very fast since the same binding inhibition was observed when the polyanions were added with the virus vs. incubation of cells with polyanions for extended period of time before virus addition.

Detailed Description Text (55):
At concentrations required to induce and maintain single-cell suspension of TN5B1-4, the three polyanions inhibited infection, but to varying degrees. The cells were infected at MOI of 0.1, 0.5, 1, 5, 10, and 20 to determine the lowest MOI for synchoronous infection.

Detailed Description Text (56):
For dextran sulfate and pentosan sulfate, viral infection was not severely inhibited for MOI.gtoreq.1. In contrast, polyvinyl sulfate inhibited viral infection even at high MOI. The order of inhibition of viral infection was polyvinyl sulfate> >pentosan sulfate>dextran sulfate. This order is different from the order of binding inhibition, indicating that inhibition of infection was not due to the reduction in binding rate. Additional evidence suggests binding is not the cause of reduced infectivity. First, dextran sulfate inhibited infection in SF21, but did not affect binding. Second, increased inhibition of viral infection in TN5B1-4 was observed by increasing the concentration of dextran sulfate above the threshold level where binding inhibition has already leveled off.

Detailed Description Text (57):
At concentrations required for single-cell formation, viral infection in TN5B1-4 was inhibited in the order: polyvinyl sulfate> >pentosan sulfate>dextran sulfate. Although these sulfated polyanions reduced virus binding to TN5B1-4, binding was still significant with the lowest binding rate still comparable to binding of virus to SF21, suggesting that inhibition of viral infection occurred at a step subsequent to binding. This hypothesis is confirmed with SF21 where dextran sulfate inhibited infection but not binding.

Detailed Description Text (58):
Different infection strategies are recommended for the various polyanions when using TN5B1-4 with the baculovirus expression vector system. For TN5B1-4 grown in dextran sulfate or pentosan sulfate, the cells can be synchronously infected in the presence of these polyanions at MOI.gtoreq.1. For polyvinyl sulfate, which blocked infection severely, the polyanion can be removed transiently by resuspending the cells in polyanion-free media or by neutralizing with a polycation such as Cat-Floc before virus addition. An equivalent amount of polyvinyl sulfate is then added back after substantial amount of virus has been internalized. In general, a virus incubation time of 2 h at MOI.gtoreq.2 before re-addition of polyvinyl sulfate should be adequate for synchronous infection at cell densities greater than 5.times.10.sup.5 cells/ml. (Table 3)

Detailed Description Text (61):
To compare recombinant protein production of cells passaged in different polyanions, cells were harvested by centrifugation at 125.times.g for 5 min, resuspended in fresh medium, and infected at 2.times.10.sup.6 cells/ml with an MOI of 5 in the presence of the polyanion dextran sulfate and pentosan sulfate. Cells were grown for at least 5 passages in a particular polyanion before infection. Polyvinyl sulfate-treated cells were infected in the absence of polyvinyl sulfate which was added back 2 h after virus addition; this protocol was necessary to bypass the inhibition of viral infection by polyvinyl sulfate as discussed above.

Detailed Description Text (71):

The enhanced protein yield was not specific to dextran sulfate-treated cells since pentosan sulfate and polyvinyl sulfate treated cells produced as well (Table 3). The secretion level for alkaline phosphatase in the three polyanions was over 80 percent at day 3 when cell viability was still over 80 percent. Enhancement is probably just from single-cell formation since dextran sulfate did not enhance protein production in suspension of SF21 cells which are already single cells.

Detailed Description Text (72):
The present invention teaches using non-carboxylated sulfated polyanions to rapidly induce and maintain single-cell suspension of TN5B1-4 cells which resulted in significant enhancement of recombinant protein production. The enhancement may be a result of the elimination of cell--cell contact which is known to severely inhibit protein synthesis in attached cultures. Improved nutrient and oxygen transport to single cells versus clumped cells could also contribute to the increased yields. Also, single cells are more accessible to virus than clumped cells.

Detailed Description Text (74):
The three most effective polyanions for single-cell formation were dextran sulfate, polyvinyl sulfate, and pentosan sulfate--all highly sulfated. Dextran sulfate is the least expensive ($1.3/g) and increases medium costs by only 3 cents/L at 25 mg/ml - not a significant cost upcharge with a medium that costs $5-30/L. The cost of dextran sulfate treatment is low compared to heparin which required a 20-fold higher level to induce single-cell suspension and would add $26/L to medium costs. More importantly, dextran sulfate does not block viral infection at MOI.gtoreq.1 whereas heparin is known to seriously inhibit infection, an attribute shared by polyvinyl sulfate.

Detailed Description Text (75):
Non-carboxylated sulfated polyanions, i.e. dextran sulfate, pentosan sulfate, and polyvinyl sulfate, have been shown herein to work well. Those skilled in the art will be able to create new compounds that also work for the methods and media described herein. For example, sulfated polysaccharides, with a degree of sulfation per monosaccharide greater than 1.5, and sulfated hydrocarbon chains should work. Furthermore, derivatives of these compounds can be made that will work as well. For example, carboxymethyl dextran benzylamide sulfonate and a co-polymer of acryllic and polyvinyl sulfate are derivatives that will probably work at a slightly greater concentration than the compounds described above. Those skilled in the art can easily test the effectiveness of these derivatives using the techniques described herein

Detailed Description Text (76):
The intrinsic advantage of TN5B1-4 cells over the commonly used SF cell lines in per cell production can now be realized using single-cell suspension of TN5B1-4 cells which eliminates the problems that have so far limited the use of T. ni cells for large-scale production, including difficulty of growing T. ni cells in suspension, the poor yield in suspension, and the inefficiency of scaling-up attached cultures. The optimum volumetric yield of SEAP in TN5B 1-4 DS 5000 cells under elevated oxygen but unsupplemented medium is 6 to 11-fold higher than attached cultures, and 3-fold higher than the best yield obtained for SF21 cells in suspension at elevated oxygen and with nutrient supplementation. The optimal per cell production of .beta.-galactosidase in TN5B1-4 cells was 350 U/10.sup.6 cells in unsupplemented medium compared to 140 U/10.sup.6 cells for SF21 cells with nutrient supplementation. The peak volumetric .beta.-galactosidase yield of TN5B1-4 cells at 2.times.10.sup.6 cells/ml in unsupplemented medium is lower compared to SF21 cells at 7.times.10.sup.6 cells/ml in supplemented medium (705 vs. 855 U/ml) probably because of nutrient limitations in TN5B1-4 cells as indicated by the rapid decline in per cell production with increasing density. By maintaining specific productivity with appropriate nutrient supplementation, TN5B1-4 cells should easily surpass the peak volumetric yield of .beta.-galactosidase in SF21 cells.

Detailed Description Text (77):
The relative ease of post-translational modifications and secretion between cell lines can be compared by considering the weight ratio of a cytoplasmic protein to a glycosylated secreted protein. For example, the weight ratio of the cytoplasmic protein b-galactosidase to the glycosylated secreted protein SEAP for TN5B 1-4 was

40-50 compared to 80-100 obtained by Taticek (1995) for SF21 cells in suspension, indicating that post-translational modifications and secretion may be more severely limited in SF21 cells than in TN5B1-4 cells. A similar difference has been observed in attached cultures where the ratio is 60 for TN5B1-4 versus 160-200 for SF9 and SF21 cells. Although we have not considered virus production in this paper, M. S. Donaldson (personal communication) has shown that the virus tilers obtained in TN5B1-4 DS 5000 cells are comparable to SF21 cells at 1-3.times.10.sup.9 PFU/ml obtained by infecting at cell densities of 1-2.times.10 .sup.6 cells/ml.

Detailed Description Text (78):
The intrinsic advantage in per cell production of TN5B1-4 over the commonly used SF cell lines can now be realized in suspension culture by using inexpensive sulfated polyanions to rapidly induce and maintain single-cell suspension of TN5B1-4 cells which substantially enhanced protein production. High yields of up to 110 mg/L of the glycosylated, secreted protein human alkaline phosphatase and 2.3 g/L of the cytoplasmic protein .beta.-galactosidase have been obtained under elevated oxygen. Nutrient supplementation might potentially increase these yields further.

Detailed Description Paragraph Table (1):
TABLE 1

Effect of Various Compounds on Inducing Single-cell Formation of TN5B1-4 Cells In Suspension Culture Polymer Conc. MW Sulfate/carboxylate Virus dissacharides (.mu.g/ml) Clumpin.sup.a (kd) per mono-saccharide .mu.eq/mg.sup.c neq/ml Binding.sup.f

A.

Polyanion dextran sulfate I 15 ++ 12.5 2.4/0 5.9 89 dextran sulfate I 25 +++ 12.5 148 42 .+-. 2 dextran sulfate II 25 ++ 100 2.4/0 5.9 148 82 .+-. 4 Pentosan sulfate 25 + 3 2/0 5.3 133 53 .+-. 3 Pentosan sulfate 100 +++ 3 2/0 530 55 .+-. 9 Heparin 100 ++ 16-17 1-1.5/0.5 6.0 600 63 .+-. 3 Heparin 270 ++ 16-17 1,620 Heparin 540 +++ 16-17 3,240 .lambda.-carrageenan 12.5 - 250-300 1-1.3/0 3.6 180 .lambda.-carrageenan 25.sup.d ++ 250-300 360 68 .+-. 3 Fucoidan sulfate 25 + 112 1/0 3.0 75 53 .+-. 1 Fucoidan sulfate 100 ++ 112 300 53 .+-. 1 Chondroitin sulfate 100 + 50 0.5/0.5 4.0 400 94 .+-. 3 Chondroitin sulfate 300 ++ 50 1,200 Dermatan sulfate 100 + 24.9 0.5/0.5 4.0 400 65 .+-. 7 Dermatan sulfate 300 ++ 24.9 1,200 .kappa.-carrageenan 100 - 150-250 0.5/0 2.3 230 73 .+-. 6 sodium CMC 1000 - 90 .sup. 0/1.sup.b 4.2 4,200 107 .+-. 1 Hyaluronate 100 - 59-104 0/0.5 2.5 250 99 .+-. 1 Polyphosphoric acid 100 - 1-2 [NaPO.sub.3].sub.n 9.8 980 98 .+-. 1 Polyphosphate 100 - 1.4-2 Na.sub.15 P.sub.13 O.sub.40 9.5 950 Na.sub.20 P.sub.18 O.sub.55 Polyvinyl sulfate 10 25 ++ +++ >100 ##STR1## 6.2 62 155 52 .+-. 4 52 .+-. 1 B. Polycation Protamine sulfate 50 - 5 virus ppt. DEAE dextran 5.sup.d - 630 74 .+-. 9 C. Neutral polymer Dextran 100 - 11.5 97 .+-. 2 Dextran 100 not tested 580 99 .+-. 3 Pluronic F68 1000 - 84 98 .+-. 2 D. Disaccharides.sup.c Heparin 100 not tested 0.6 1-1.5/0.5 6.0 600 103 .+-. 2 Chondroitin sulfate 100 - 0.5 0.5/0.5 4.0 400 99 .+-. 6

.sup.a +++ Single cells; ++ single cells plus few clumps of 10 cells maximum; + slight improvement over control; - no improvement. .sup.b Estimated; maximum theoretical substitution is 3 but average commercial substitution is generally less than 1.5. .sup.c Calculated from sulfur content if available or else from formula. .sup.d Toxic above these levels. .sup.e Enzymatic digests of the polysaccharides. .sup.f For 30 min as a % of ControlControl refers to binding of virus to cells with no additive.

Detailed Description Paragraph Table (2):
TABLE 2 Effect of Sulfated Polyanions on AcMNPV Binding to TN5B1-4 Cells Concentration Binding (.mu.g/ml) (% of control)

A. Binding in Presence of Polyanions.sup.a

Dextran Sulfate 25 42 .+-. 2 Polyvinyl Sulfate 25 52 .+-. 4 Pentosan Sulfate 100 55 .+-. 9 B. Pretreatment of Virus.sup.b Dextran Sulfate 25 103 .+-. 3 Polyvinyl Sulfate 25 96 .+-. 5 Pentosan Sulfate 100 99 .+-. 4 C. Pretreatment of Cells.sup.c Dextran Sulfate 25 93 .+-. 9 Polyvinyl Sulfate 25 95 .+-. 6 Pentosan Sulfate 100 96 .+-. 7

.sup.a Binding at 5 .times. 10.sup.6 cells/ml for 30 min; control is binding in absence of polyanion. .sup.b Virus pretreated with polyanion for 2 h, pelleted at 13,000 .times g for 1 h, resuspended in PBS, and allowed to bind to 5 .times. 10.sup.6 cells/ml for 1 h without further polymer addition; control is untreated virus. .sup.c Cells pretreated with polyanion

for 3 h, washed once in ExCell 405 and allowed to bind to 5 .times. 10.sup.6 cells/ml for 1 h without furthe polymer addition; control is untreated cells.

Detailed Description Paragraph Table (3):
TABLE 3

Secreted Alkaline Phosphatase Expression using Different Infection Strategies (Ex-Cell 405, MOI = 5)* Gas-Phase % Secreted % Viability Total SEAP Polyanion (25 mg/ml) O.sub.2 Infection Strategy Day 3 Day 3 Day 5 (U/ml

A. 2 .times. 10.sup.6 cells/ml Clumped control 21 virus added directly to cells -- >60 3 .+-. 2 Dextran Sulfate (DS) 21 in presence of DS 83 .+-. 2 83 .+-. 2 42 .+-. 4 Pentosan Sulfate (PS) 21 in presence of PS 90 .+-. 4 82 .+-. 2 41 .+-. 5 Polyvinyl Sulfate 21 strategy 1 87 .+-. 2 81 .+-. 1 45 .+-. 7 (PVS) Polyvinyl Sulfate 21 strategy 2 89 .+-. 4 83 .+-. 2 43 .+-. 5 B. 5 .times. 10.sup.6 cells/ml Clumped control 80 virus added directly to cells -- -- 15.sup.+ Dextran Sulfate 80 in presence of DS 85 .+-. 3 72 .+-. 2 68 .+-. 4 Polyvinyl Sulfate 80 strategy 2 89 .+-. 4 65 .+-. 3 64 .+-. 2

*Cells grown in polyanion for at least 5 passages before infecting in 50m spinner flasks. DS and PS cells were resuspended in fresh medium containing the polyanion before virus addition. Strategy 1: cells resuspended in polyanionfree medium, incubated with virus for 2 h before adding PVS back. Strategy 2: cells resuspended in PVScontaining medium, neutralized with CatFloc for 3 min, incubated with virus for 2 h before adding PVS back. .sup.+ From data of Taticek (1995).sup.23 at an MOI of 10

Other Reference Publication (1):
Wu, J. Et al, 1990, "Adaptation of Insect cells to suspension culture", J. Ferment. Bioeng. 70: 90-93.

Other Reference Publication (2):
Ogonah, O., et al, 1991, "Protein production (B-galactosidase) from a baculovirus vector in Spodoptera frugiperada and Trichopulsia ni cells in suspension culture", Biotech. Lett. 13: 265-270.

Other Reference Publication (5):
Taticek, R., 1995, "Enhanced recombinant protein expression in baculovirus-infected high-density insect cell suspension cultures and the operation of a continuous flow bioreactor"., Ph.D. thesis, Cornell Univ. Ithaca, NY.

Other Reference Publication (9):
Carroll et al, 1982, "Heparin-binding Agglutinin On Human Teratocarcinoma Cells: Biochem. Biophys. Res. Commun.", 109/4, pp. 1353-1359.

Other Reference Publication (10):
Gill et al, 1986, Heparin-Induced Aggregation of Lymphoid Cells, J. Cell. Physiol., 126/3, pp. 352-358.

Other Reference Publication (11):
Moore et al, Heparin-induced Agglutination of Erythrocytes in Horses, Amer. Jnl. Of Veterinary Res., 48 (1).

Other Reference Publication (12):
Mahaffey et al, 1986, Erythrocyte Agglutination Associated With Heparin Treatment In Three Horses, Jn. Amer. Veterinary Medical Assoc., 189 (11): 1478-1480.

CLAIMS:

1. A method of increasing single cell suspension in an insect cell line comprising the steps of:

b) adding an effective concentration of a non-carboxylated sulfated polyanion to said medium such that said cells are in suspension after the addition of said non-carboxylated sulfated polyanion; and

c) subculturing said insect cells which are in suspension in a fresh culture medium

including an effective concentration of said non-carboxylated sulfated polyanion.

4. The method of claim 1 in which said culture medium of step c) of claim 1 is serum free.

ii) pentosan sulfate,

7. A culture of insect cells in a culture medium including a non-carboxylated sulfated polyanion, wherein said insect cells have been induced to single cell suspension by the method of claim 1, and wherein the non-carboxylated sulfated polyanion is present in an amount effective to carry out the method of claim 1.

8. A method of increasing single cell suspension in an insect cell line comprising the steps of:

b) adding an effective concentration of a sulfated polyanion to said medium such that said cells are in suspension after the addition of said sulfated polyanion, wherein said sulfated polyanion is selected from the group consisting of:

ii) pentosan sulfate,

c) subculturing said insect cells which are in suspension in a fresh culture medium including an effective concentration of said non-carboxylated sulfated polyanion.

11. The method of claim 8 in which said culture medium of step c) of claim 8 is serum free.

12. A culture of insect cells in a culture medium including a non-carboxylated sulfated polyanion, wherein said insect cells have been induced to single cell suspension by the method of claim 8, and wherein the non-carboxylated sulfated polyanion is present in an amount effective to carry out the method of claim 8.